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Silencing of the Tapetum-Specific Zinc Finger Gene *TAZ1* Causes Premature Degeneration of Tapetum and Pollen Abortion in Petunia

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***TAZ1* (*TAPETUM DEVELOPMENT ZINC FINGER PROTEIN1*; renamed from *PEThy; ZPT3-2*) cDNA was first isolated as an anther-specific cDNA from petunia. Here, we report a functional characterization that includes analysis of spatial and temporal expression profiles and examination of anther phenotypes in *TAZ1*-silenced plants. *TAZ1* showed a biphasic expression pattern. In the premeiotic phase, *TAZ1* transcripts were found to accumulate in all cell types of the anther except the tapetum and gametophytic tissues, whereas the postmeiotic phase of anther development was characterized by expression exclusively in the tapetum. Silencing of *TAZ1* by cosuppression resulted in aberrant development and precocious degeneration of the tapetum, followed by extensive microspore abortion that started soon after their release from pollen tetrads. A few pollen grains that survived showed reduced flavonol accumulation, defects in pollen wall formation, and poor germination rates. This study demonstrates an essential role for *TAZ1* in the postmeiotic phase of tapetum development.**

INTRODUCTION

Development of the male gametophyte (pollen) occurs within the anther compartment of the stamen and requires cooperative functional interactions between gametophytic and sporophytic tissues (Raghavan, 1997a). The tapetum is the innermost of the four sporophytic layers of the anther wall that comes in direct contact with the developing gametophyte; therefore, it has long been considered to play a crucial role in the maturation of microspores (Shivanna et al., 1997). Early studies using microscopic and cytological techniques revealed that tapetal cells contain dense cytoplasm with abundant nuclei and other cell organelles, indicating the metabolically dynamic state of these cells (Stevens and Murray, 1981). The plasma membrane facing the developing microspores in these cells was shown to develop membrane-lined lipid bodies (orbicules), which were thought to contribute to the synthesis of the pollen wall (Steer, 1977). Additionally, a number of male-sterile mutants were found to have defects in the structure of the tapetum (Raghavan, 1997b). The indispensability of the tapetum during microspore development was demonstrated further by the specific ablation of tapetal cells by targeted expression of cytotoxin genes, which resulted in the premature abortion of microspores (Goldberg et al., 1993).

The understanding of the function of the tapetum prompted the identification of tapetum-specific genes such as those coding for callase (Hird et al., 1993) and the enzymes involved in the biosynthesis of lipids (Foster et al., 1992) and flavonoids (Koes et al., 1990; van Tunen et al., 1990). Genes coding for oleosins, which are specialized proteins that enclose naked oil droplets of storage triglycerides, and those for small Gly- or Cys-rich secretory proteins that expressed specifically or preferentially in the tapetum, also were characterized (Aguirre and Smith, 1993; Chen and Smith, 1993; Robert et al., 1994; Ross and Murphy, 1996; Ruiter et al., 1997). Regarding the hierarchy of gene function during flower development, the examples cited above belong to the category of downstream genes that are required for the function of the tapetum. On the other end of this regulatory hierarchy, a number of "master" regulatory genes that are involved in the establishment of the floral meristem and the determination of floral organ identity also have been characterized (reviewed by Theiben, 2001). The most neglected aspect of flower development, however, has been the delineation of the middle-level regulatory mechanisms

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that commence after floral organ primordia have been established and direct the completion of organ differentiation (Smyth, 2001).

We previously identified a number of genes for floral organ–specific zinc finger transcription factors in petunia by PCR-based screening of organ-specific cDNA libraries (Takatsuji, 1999). Seven such anther-specific genes were found to express sequentially but transiently during the course of anther development, suggesting that they might form a regulatory cascade to control anther development (Kobayashi et al., 1998). Of these, the expression peak of *TAZ1* (*TAPETUM DEVELOPMENT ZINC FINGER PROTEIN1*; renamed from *PEThy-ZPT3-2*), which encodes a protein with three zinc finger motifs, preceded those of six other genes during the early stages of anther development (Kobayashi et al., 1998). Here, we report detailed expression profiles and the functional characterization of *TAZ1*. The data show a distinctive biphasic expression pattern of *TAZ1* during the early stages of anther development in petunia. Analysis of transgenic plants, in which the *TAZ1* gene was silenced by cosuppression, demonstrated that *TAZ1* activity is essential for the postmeiotic phase of tapetum development, which in turn plays an essential role in microspore maturation. Based on these data, a possible role for *TAZ1* in the transcriptional regulation associated with the development and function of the tapetum as well as the maturation of pollen is discussed.

RESULTS

Temporal and Spatial Expression Pattern of *TAZ1* in Sporophytic Anther Tissues

To determine the temporal expression of *TAZ1*, RNA gel blot analysis was performed using anthers from 12 early (bud lengths [Bl] 3 to14 mm at 1-mm intervals) and 5 late (Bl 15 to 20, 20 to 30, 30 to 40, 40 to 50, and 50 to 60 mm) stages of flower development. The early stages up to Bl 14 mm displayed *TAZ1* expression ranging from strong (Bl 3 to 4 mm) to medium (Bl 5 to 8 mm) to low (Bl 9 to 14 mm) (Figure 1). Bl 3 to 4 mm corresponds to the pollen mother cell stage of microspore development, which is followed by meiosis in Bl 4 to 5 mm. The uninucleate stage of microspore development extends from Bl 6 to 20 mm. Meanwhile, the tapetum reaches the peak of its development by Bl 6 to 8 mm; thereafter, it gradually starts to degenerate. By Bl 35 mm, the tapetum has disappeared completely from the anthers. Notably, the disappearance of *TAZ1* mRNA coincided well with the degeneration of the tapetum.

To define the cell type–specific expression pattern of *TAZ1*, in situ hybridization analysis was performed on transverse sections of 3-, 4-, 5-, 6-, 7-, 9-, and 13-mm flower buds. The entire *TAZ1* cDNA was used to prepare sense and antisense RNA probes, as described in Methods. This

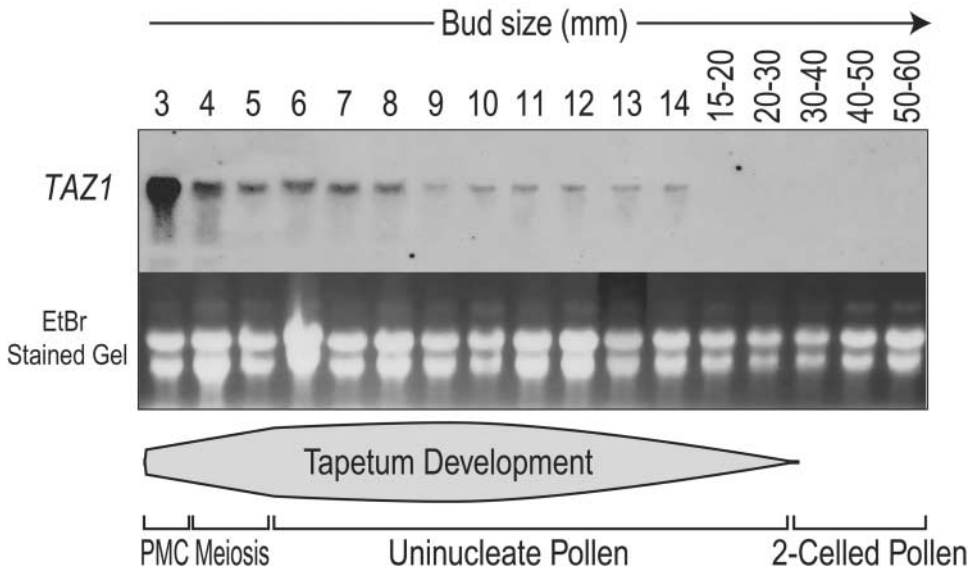

Figure 1. Temporal Expression Pattern of *TAZ1*.

RNA gel blot analysis using total anther RNA from wild-type plants and antisense digoxigenin-labeled *TAZ1* RNA as a probe. The numbers at top of each lane represent the length of flower buds in millimeters. A graphic representation of tapetum and pollen development corresponding to the size of flower buds is displayed at bottom. EtBr, ethidium bromide; PMC, pollen mother cells.

analysis revealed two distinct phases of *TAZ1* expression (Figure 2). In the early developmental stages up to Bl 5 mm (i.e., phase I), *TAZ1* expression was detected in all of the cell types of anther except tapetum and the sporogenous tissue (Figures 2A to 2I). In sharp contrast to this, during phase II (Bl 6 to 13 mm), the *TAZ1* mRNAs were detected exclusively in the tapetum (Figures 2J to 2U).

In the early stages of phase I, *TAZ1* expression was detected uniformly in the connective, vascular tissue, wall layers, and stomium cells (Figures 2B and 2C). However, with the progression of development, it gradually started to concentrate in the stomium and the connective cells adjoining the inner tapetum (Figures 2H and 2I). Most notably, soon after the separation of individual microspores from tetrads in Bl 6 mm, there was a dramatic shift in the *TAZ1* expression pattern that marked the beginning of phase II (Figures 2J to 2U). In this phase, expression was localized exclusively in the tapetum, whereas in the surrounding tissues, it was reduced to almost undetectable levels. In tapetal cells, the high levels of *TAZ1* expression continued at least until Bl 8 mm (Figures 2Q and 2R), and as the anther matured, there was an overall decrease in the expression levels that coincided with the degeneration of the tapetum. In agreement with the RNA gel blot data (Figure 1), transverse sections of anthers from Bl 13 mm showed significant reductions in *TAZ1* expression (Figures 2T and 2U). Collectively, these data demonstrate two distinct phases in which *TAZ1* expression is regulated in a spatial and temporal manner.

A 2-kb Upstream Region Is Sufficient for the Phase II–Specific Expression of *TAZ1*

To delineate the promoter region of *TAZ1*, we constructed transgenic petunia plants with a chimeric gene containing a β-glucuronidase (GUS) coding sequence fused to an ~2-kb upstream region of *TAZ1*. Five independently transformed lines were selected for the characterization of the promoter activity. GUS expression was localized mainly in the anthers of young flower buds in these lines (Figure 3A). Further histochemical analysis localized the GUS expression mostly in the tapetal cells; however, a low level of GUS activity was detected in the adjoining cells of the middle layer and in the connective (Figures 3B and 3C). In contrast to the RNA gel blot and in situ hybridization analyses, which displayed relatively higher *TAZ1* expression during phase I, GUS activity was undetectable in phase I flower buds (Bl < 6 mm; data not shown). The absence of GUS activity in young buds may suggest that (1) the *cis* element responsible for *TAZ1* expression in the premeiotic phase existed either farther upstream of the 2-kb promoter region or downstream of the translation initiation site, or (2) the 271-bp *TAZ1* 5′ untranslated region (Figure 3D) contained some regulatory sequence(s) that suppressed the translation of the transcripts during phase I.

TAZ1-GFP Is Targeted to the Nucleus

The presence of three transcription factor IIIA (TFIIIA)-type zinc finger motifs strongly suggested that *TAZ1* is a transcription factor. To qualify as a transcription factor, however, a protein must be translocated into the nucleus to interact with its target DNA. TAZ1 contains two basic motifs, 5′-KKCKKLNPFGSRYYKKR-3′ (amino acids 81 to 99) and 5′-KKKKKK-3′ (amino acids 263 to 270), resembling the bipartite and the monopartite nuclear localization signals, respectively. Based on the presence of these basic motifs, it was predicted to be a nucleus-localized protein with a certainty of 0.96 by PSORT (www.psort.nibb.ac.jp). To determine whether TAZ1 is imported into the nucleus in vivo, onion peel cells were bombarded with *35S-sGFP-TAZ1* and a control plasmid (*35S-sGFP*) (Figure 4B). In the case of the control plasmid, the green fluorescent protein (GFP) was distributed throughout the cytoplasm and the nucleus (Figure 4A, left). By contrast, the recombinant protein sGFP-TAZ1 was localized exclusively in the nucleus (Figure 4A, right). These data indicate that TAZ1 is a nuclear protein that probably interacts with the DNA and functions as a transcription factor.

Functional Analysis of *TAZ1* Using *TAZ1*-Silenced Plants

The complete *TAZ1* cDNA (*cTAZ1*) under the control of the 35S promoter of *Cauliflower mosaic virus* (*CaMV 35S* promoter) was introduced into petunia by *Agrobacterium tumefaciens*–mediated transformation (Figure 5A). The accumulation of *TAZ1* transcripts in transgenic plants was analyzed by gel blot analysis of the total RNA extracted from leaves of 15 transformants. Six transgenic lines (1, 10, 12, 16, 17, and 19) showed moderate to high accumulation of *TAZ1* transcripts in leaves (Figure 5B). However, they did not display any apparent physiological or morphological aberrancy under normal greenhouse conditions; therefore, they were not analyzed further. The transgenic lines that did not express *TAZ1* RNA in leaves were analyzed for its expression in anthers. The RNA samples were prepared from pooled anthers from Bl 3 to 6 mm (two buds at each 1-mm interval), representing both phase I and phase II of anther development. Of the nine transgenic lines that did not show *TAZ1* expression in leaves, five (2, 8, 13, 14, and 20) accumulated *TAZ1* RNA in anthers at levels similar to that in the wild type and were morphologically and developmentally indistinguishable from the wild type. The rest of the transgenic lines (5, 9, 15, and 18), however, did not show any *TAZ1* expression in anthers, suggesting cosuppression of *TAZ1* gene expression (Figure 5C). Because the RNA preparations contained representative anthers from both phase I and phase II (described above), it is possible that *TAZ1* expression was silenced in all tissues of the anther, including the tapetum, in these four transgenic lines.

The *CaMV 35S* promoter has been shown to be expressed in the vascular tissue, connective, and wall layers

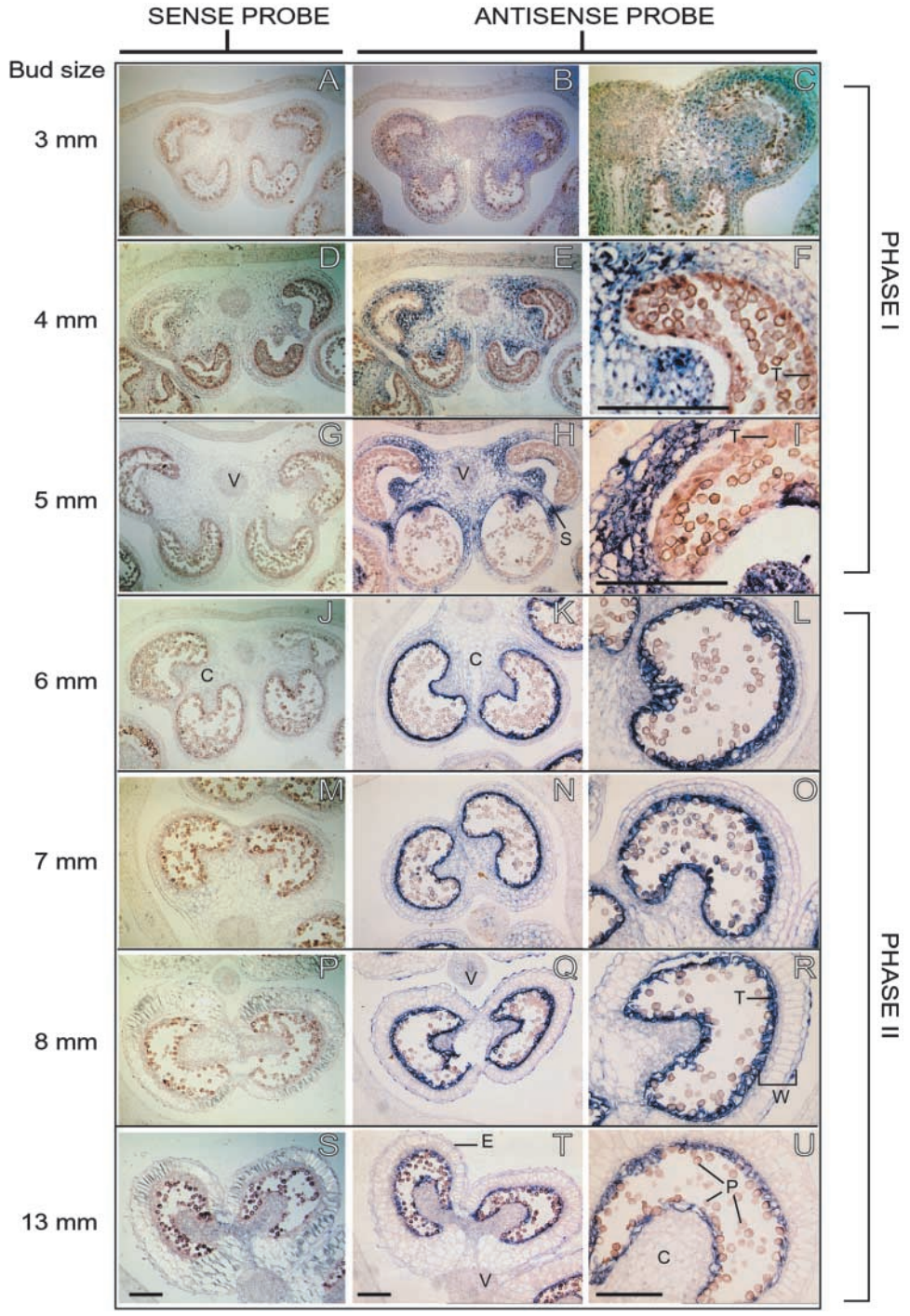

Figure 2. Analysis of the Spatial and Temporal Expression Patterns of *TAZ1* by in Situ Hybridization.

Transverse sections of anthers from early stages of development (as indicated at left) were probed with sense (**[A]**, **[D]**, **[G]**, **[J]**, **[M]**, **[P]**, and **[S]**) and antisense (**[B]**, **[E]**, **[H]**, **[K]**, **[N]**, **[Q]**, and **[T]**) *TAZ1* probes. **(C)**, **(L)**, **(O)**, **(R)**, and **(U)** at right represent ×2-magnified views of the corresponding images in the middle column. **(F)** and **(I)** at right represent ×4-magnified views of the corresponding images in the middle column. C, connective; E, epidermis; P, pollen; S, stomium; T, tapetum; V, vascular bundle; W, wall layers. Bars = 200 μm.

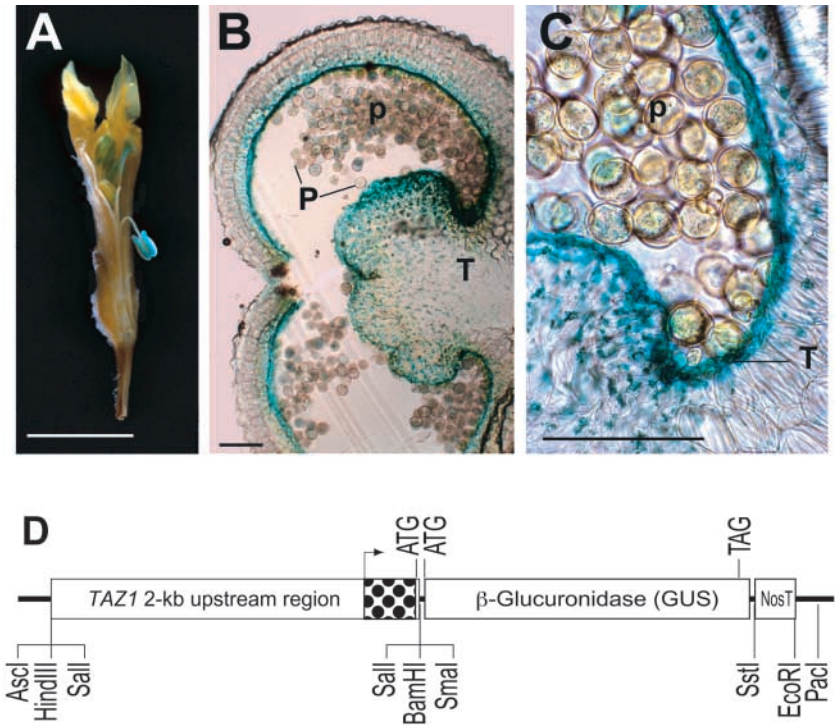

Figure 3. *TAZ1* Promoter-Driven GUS Expression in Transgenic Plants.

(A) to **(C)** GUS staining of a 25-mm flower bud **(A)** and transverse sections through the anther (**[B]** and **[C]**) of a *TAZ1*::*GUS* transgenic plant. P, pollen; T, tapetum. Bar in **(A)** = 5 mm; bars in **(B)** and **(C)** = 100 μm.

(D) Scheme of a *TAZ1*::*GUS* construct. The transcription initiation site is marked with a bent arrow. The translation initiation and termination sites are shown as ATG and TAG, respectively, and the *TAZ1* 5′ untranslated region is shown with a dotted pattern.

but not in the tapetum (Plegt and Bino, 1989; van der Meer et al., 1992). Therefore, it is unlikely that *TAZ1* expression under the control of the *CaMV 35S* promoter could have triggered cosuppression directly in the tapetal cells. Because the silencing signal is known to propagate through the plant (Vaucheret et al., 1998), one can postulate that a *TAZ1* silencing signal translocated from the site of its initiation, presumably from the cells of the connective and/or anther wall, to the adjoining cells of the tapetum. In these lines, *TAZ1* transgene expression was not detected even in leaves.

To verify the specificity of *TAZ1* silencing, we analyzed the expression of another zinc finger–encoding gene, *ZPT2-5*, in *TAZ1*-silenced lines (Figure 5D). Of the seven anther-specific zinc finger genes described previously, the expression profile of only *ZPT2-5* overlapped with that of *TAZ1*, whereas others were expressed later during anther development (Kobayashi et al., 1998). Furthermore, the conserved zinc finger–encoding regions in *TAZ1* and *ZPT2-5* displayed the highest level (~68%) of sequence similarity among all of the anther-specific zinc finger genes. The level of *ZPT2-5* transcripts in *TAZ1*-silenced lines was the same as that in the wild type, confirming that the silencing was specific to *TAZ1*. These plants had extremely low pollen counts at the time of dehiscence. Apparently, however, there was no effect on female fertility, because cross-pollination of *TAZ1*-silenced flowers by wild-type pollen yielded normal seed set.

Development of the Tapetum in *TAZ1*-Silenced Plants

Transverse sections of anthers from wild-type and *TAZ1*-silenced plants were analyzed for the effect of *TAZ1* silencing on various cell types of the anther (Figure 6). Early stages of anther development (phase I) showed no noticeable effect on the development of the wall layers, connective, vascular tissue, and stomium cells. In wild-type plants, tapetum in the anthers at Bl 4 mm consists of a single layer of columnar cells (Figure 6A). Thereafter, the tapetum undergoes a phase of active cell proliferation to become multilayered by the time individual microspores are released from pollen tetrads (Bl 6 mm; Figure 6C). At this stage, the tapetum cells

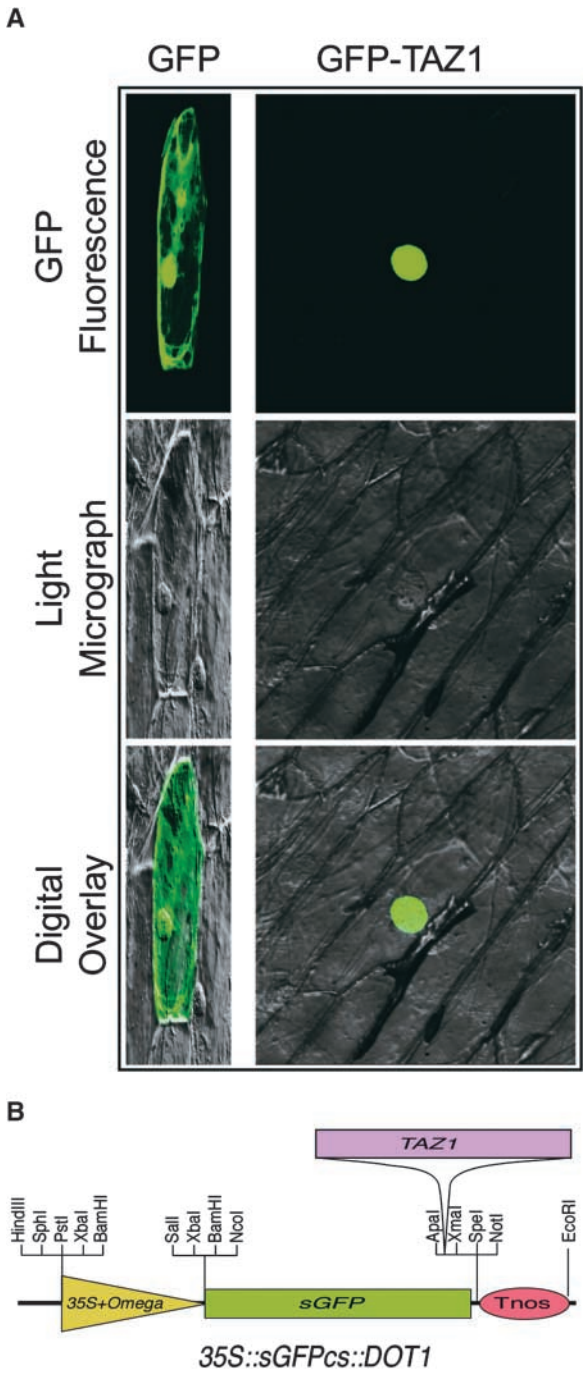

Figure 4. TAZ1 Is a Nucleus-Localized Protein.

(A) DNA constructs encoding sGFP (left) and the chimeric sGFP-TAZ1 (right) proteins were bombarded into onion peel cells, and the subcellular localization of fusion proteins was observed by GFP visualization with a confocal microscope.
(B) Scheme of the *35S*::*sGFPcs*::*TAZ1* construct.

are characterized by a dense cytoplasm and a few small vacuoles. By Bl 9 mm, the tapetum is reduced to two layers, but the size of individual tapetal cells increases significantly (Figure 6E). The gain in cell size continues until the Bl 13-mm stage, concomitant with the degeneration of inner tape-

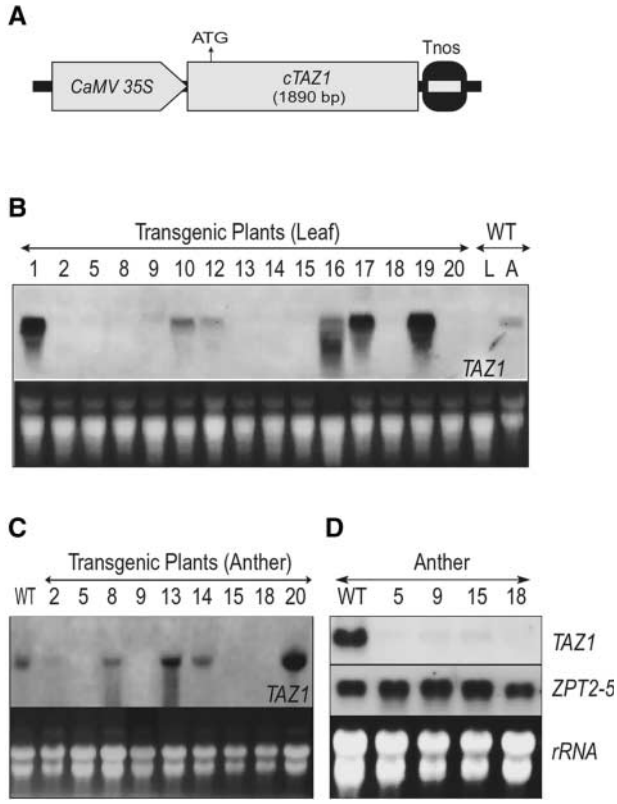

Figure 5. Analysis of *TAZ1* Transcripts in *CaMV35S*::*TAZ1* Transgenic Petunia Plants.

(A) The *35S*::*TAZ1* construct. The 1890-bp *cTAZ1* was inserted between the *CaMV 35S* promoter (800 bp) and the nopaline synthase terminator (Tnos). The translation initiation site of TAZ1 is marked as ATG.
(B) RNA gel blot analysis using total leaf RNA and antisense digoxigenin-labeled *TAZ1* RNA as a probe. The numbers at top of each lane represent individual transgenic lines, and the last two lanes contained total RNA from leaves (L) and anthers (A) of wild-type plants.
(C) RNA gel blot analysis using total anther RNA and antisense digoxigenin-labeled *TAZ1* RNA as a probe of those transgenic lines that did not show *TAZ1* expression in leaves **(B)**.
(D) Specific silencing of *TAZ1*. Total RNA samples from the anthers of wild-type and transgenic plants (lanes 5, 9, 15, and 18) were probed with antisense digoxigenin-labeled *TAZ1* (top) and *ZPT2-5* (middle) RNA as probes.
The bottom gels in **(B)** to **(D)** show ethidium bromide–stained RNA gels photographed before blotting. The 26S RNA (top bands) in **(B)** is eclipsed by comigrating bromphenol blue. WT, wild type.

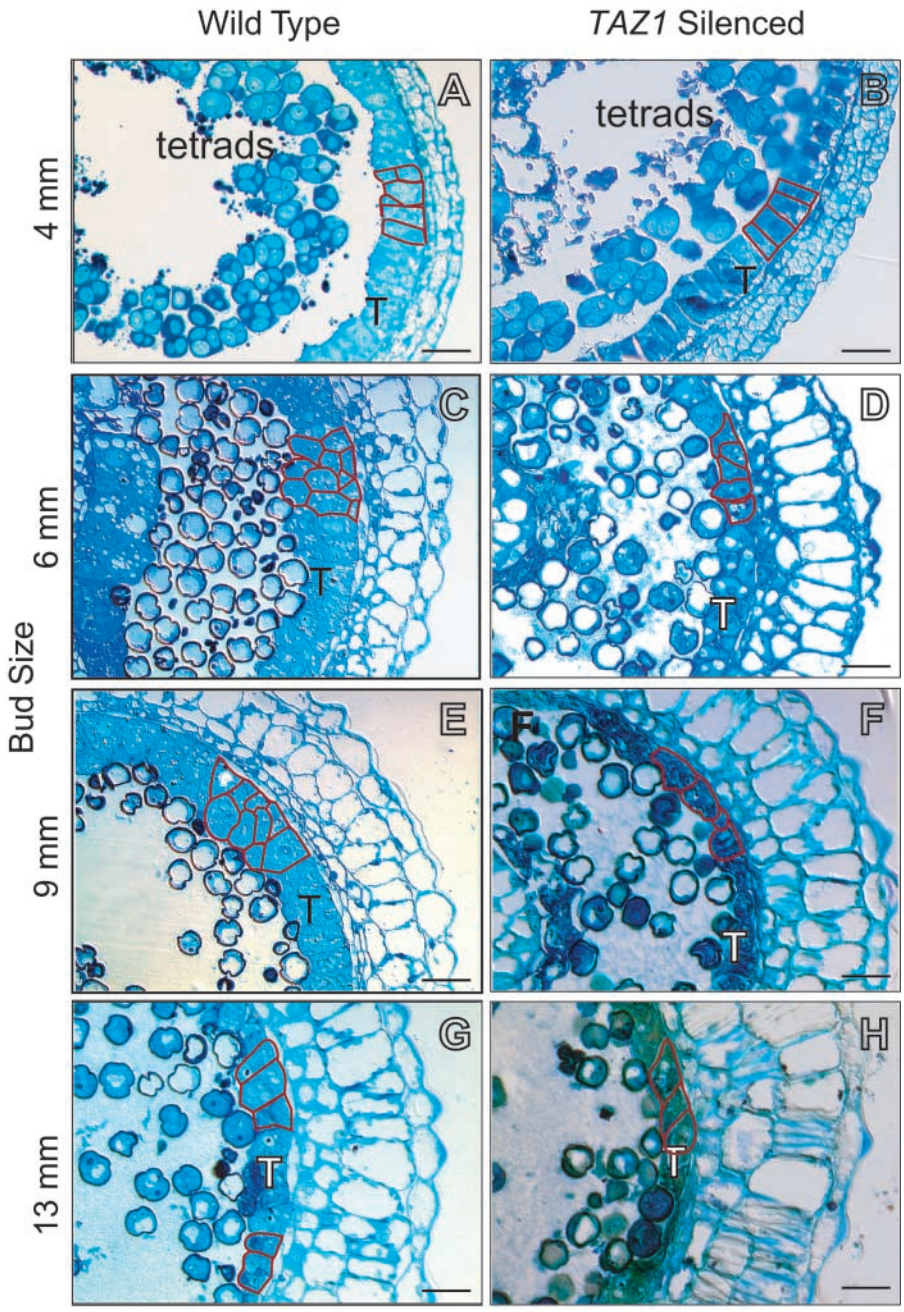

Figure 6. Silencing of *TAZ1* Results in Poorly Developed Tapetum.

Transverse sections through wild-type (**[A]**, **[C]**, **[E]**, and **[G]**) and *TAZ1*-silenced (**[B]**, **[D]**, **[F]**, and **[H]**) anthers during early stages (4- to 13-mm bud size) of flower development. In each image, a few tapetal cells are outlined in red. T, tapetum. Bars = 50 μm.

tum layers. At the Bl 13-mm stage, the tapetum is left with only a single layer of cells with less dense, vacuolated cytoplasm (Figure 6G). The degeneration process continues and the tapetum disappears completely by the time the flower buds are 35 mm in size (data not shown).

In *TAZ1*-silenced lines, the development of tapetum at the Bl 4-mm stage was comparable to that in the wild type in having a monoseriate tapetum at the completion of meiosis (Figure 6B). However, soon after the separation of individual haploid microspores from tetrads, the tapetal cells

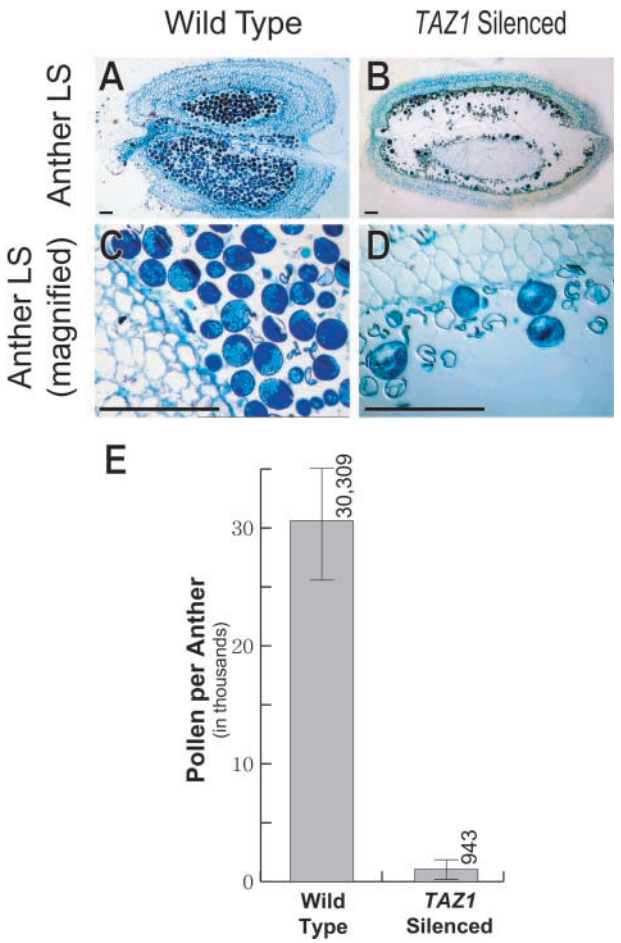

Figure 7. Pollen Degeneration as a Result of *TAZ1* Silencing.

(A) and **(B)** Longitudinal sections of mature anthers from 65-mm buds of wild-type and *TAZ1*-silenced plants.

(C) and **(D)** Enlarged views of **(A)** and **(B)**, respectively. Bars = 100 μm.

(E) Number of intact pollen grains in wild-type and *TAZ1*-silenced plants. The numbers at top of each bar represent the average intact pollen count of six individual anthers from three different flower buds. Error bars represent standard deviations.

started to degenerate. Noticeably, at the BI 6-mm stage, the tapetum in *TAZ1*-silenced lines consisted of a single layer of vacuolated cells, instead of the multilayered tapetum that was observed in wild-type anthers at this stage. The extent of tapetum degeneration at this stage in *TAZ1*-silenced anthers was comparable to that of the BI 13-mm stage in wild-type plants (Figures 6D and 6G). The degeneration of tapetal cells continued during the BI 9-mm stage, and most of the cells had collapsed by the BI 13-mm stage (Figures 6F and 6H).

Effects of *TAZ1* Silencing on Pollen Development

Premature Pollen Abortion

The *TAZ1*-silenced plants had drastically reduced numbers of pollen (~1000/anther) compared with wild-type plants (~30,000/anther) (Figure 7). Moreover, the surviving pollen grains in these lines were found to be fragile, because most of them burst within 10 min of incubation in 10% glycerol solution (data not shown). In this solution, wild-type pollen grains remained intact for >24 h.

Poor Viability

To examine the viability of the surviving pollen in *TAZ1*-silenced lines, they were germinated under in vitro and semi-in vivo conditions, as described in Methods. As shown in Figure 8, wild-type pollen demonstrated nearly 100% germination under both conditions. Most of the pollen tubes grew ~400 μm under in vitro conditions and rarely showed bursting (Figure 6A). Under semi-in vivo conditions, the pollen tubes grew longer than 1 cm in 14 h (Figure 8C). By contrast, pollen grains from *TAZ1*-silenced lines rarely germinated. In the small fraction that germinated, the pollen tubes did not grow >80 μm in length (Figure 8B). Furthermore, most of these pollen tubes displayed burst tips (Figure 8B, inset). Under semi-in vivo germination conditions, the pollen grains from *TAZ1*-silenced lines showed poor germination, because few pollen tubes were visible at the cut end of the style (Figure 8D).

Reduced Flavonol Accumulation

The flavonols are synthesized in tapetal cells and are released subsequently into the anther locule to be taken up by the developing microspores (Mo et al., 1992; Ylstra et al., 1994). To determine whether precocious degeneration of the tapetum had any effect on the supply of flavonols to the developing microspores, both young and mature pollen grains from wild-type and *TAZ1*-silenced plants were stained for flavonols. In microspore tetrads (Figures 9A and 9B), there was no apparent difference in the accumulation of flavonols between the wild-type and *TAZ1*-silenced lines (Figures 9A and 9B). However, mature pollen grains in *TAZ1*-silenced lines displayed markedly reduced (~30-fold less) fluorescence of flavonols compared with that in the wild type (Figures 9C to 9F). Furthermore, the collapsed pollen grains in *TAZ1*-silenced lines fluoresced blue under UV light, suggesting that the walls of these microspores did not accumulate any flavonols. To determine whether flavonols generally were absent in the pollen wall or that this absence resulted from the silencing of *TAZ1* expression, intact pollen grains from wild-type and *TAZ1*-silenced lines were ruptured me-

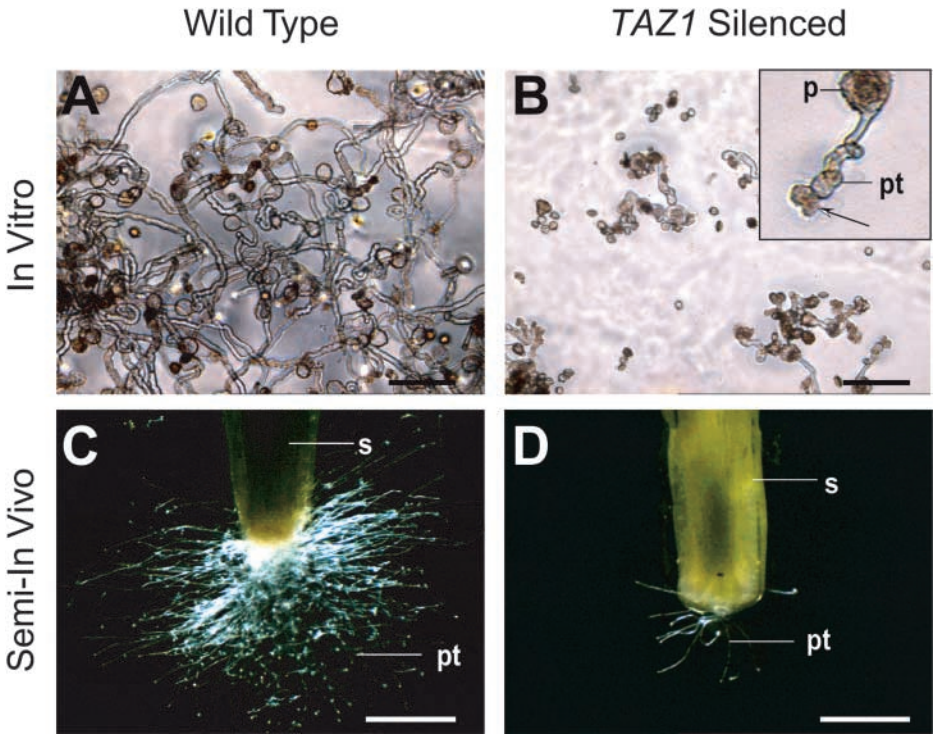

Figure 8. Effects of *TAZ1* Silencing on Pollen Germination.

Pollen grains from wild-type (**[A]** and **[C]**) and *TAZ1*-silenced (**[B]** and **[D]**) lines were germinated under in vitro (**[A]** and **[B]**) or semi-in vitro (**[C]** and **[D]**) conditions, as described in Methods. Pollen tube growth was examined after 14 h of incubation. The inset in **(B)** shows a burst pollen tube. p, pollen; pt, pollen tube; s, style. Bars = 100 μm in **(A)** and **(B)** and 400 μm in **(C)** and **(D)**.

chanically, washed, and stained for flavonols. Like the naturally collapsed pollen, intentionally ruptured pollen showed very little flavonol accumulation in *TAZ1*-silenced lines. By contrast, the walls of wild-type pollen displayed significant accumulation of flavonols (Figures 9G and 9H). Together, these data indicate that precocious degeneration of the tapetum resulting from the silencing of *TAZ1* has adverse effects on the synthesis of flavonols and/or their transport into the developing microspores and that these effects manifest themselves only after the completion of male meiosis.

Anomalies in Pollen Wall Development

To search for defects in pollen wall formation, mature pollen grains were analyzed using a scanning electron microscope. Wild-type pollen grains were spherical and had sculptured exine walls. There were three germination pores through which future pollen tubes could emerge (Figure 10A). The surviving pollen grains in *TAZ1*-silenced lines had normal-appearing exine sculpturing, yet the wall formation, especially around the germination pores, was incomplete (Figure 10B). The diameter of these pollen grains was up to 30% larger than that of the wild type, and they displayed characteristically bulging germination pores.

All plant cells, including pollen grains, undergo plasmolysis when placed in hypertonic solutions. Wild-type mature pollen grains showed characteristic separation of the plasma membrane from the pollen wall (consisting of exine and intine) when incubated in 20% glycerol solution (Figures 10C and 10E). At this concentration of glycerol, however, there was no effect on the pollen grains of *TAZ1*-silenced lines (data not shown). Even when the glycerol concentration was increased to 50%, these pollen grains remained intact (Figure 10D). However, after 2 h of incubation in this solution, the intine started to separate from the exine near the germination pores and appeared to be pulled inside, albeit slightly, along with the plasma membrane (Figures 10D and 10F). The inability of the intine to separate from the exine under strong osmotic pressure indicates abnormalities during the synthesis of the intine and/or the plasma membrane. Because precursors of pollen wall are provided mainly by the tapetum, this phenotype probably is attributable to premature degeneration of the tapetal cells.

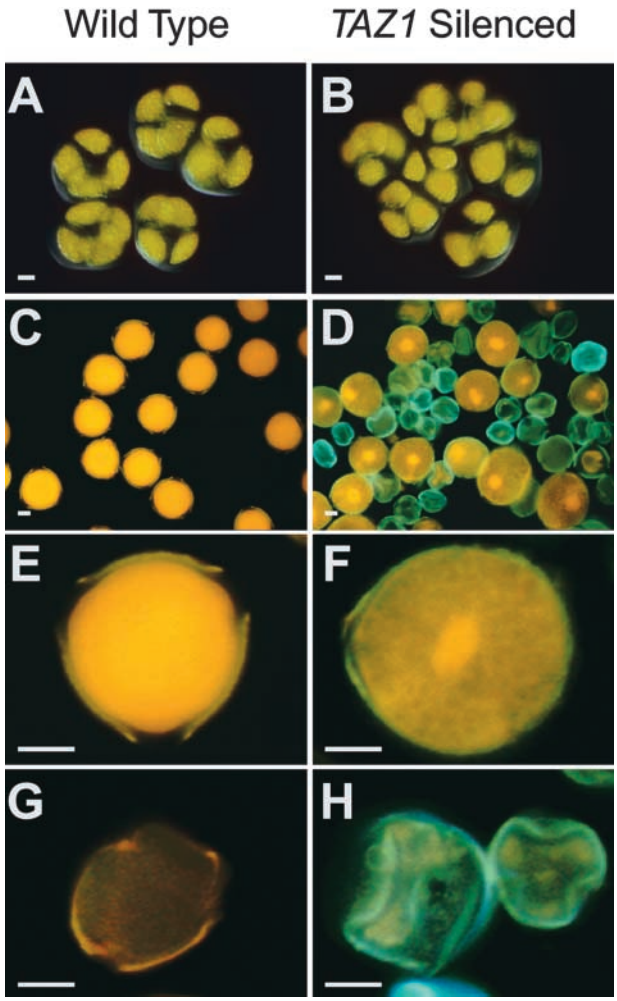

Figure 9. *TAZ1* Silencing Results in Flavonol-Deficient Pollen.

Pollen grains from young (6-mm) and mature (65-mm) flower buds of wild-type (left) and *TAZ1*-silenced (right) plants were stained for flavonols and observed under UV light.
(A) and **(B)** Pollen tetrads from 6-mm buds.
(C) and **(D)** Mature pollen from 65-mm buds.
(E) and **(F)** Single intact pollen.
(G) and **(H)** Pollen wall remains after mechanical rupturing.
Bars = 10 μm.

DISCUSSION

TAZ1 belongs to the 5-enolpyruvylshikimate-3-phosphate synthase promoter binding factor family of TFIIIA-type zinc finger transcription factors, in which two Cys and two His residues tetrahedrally coordinate a zinc atom to form a compact structure that interacts with the major groove of DNA in a sequence-specific manner (Takatsuji, 1996). The 5-enolpyruvylshikimate-3-phosphate synthase promoter binding factor family differs from the animal TFIIIA-type zinc finger protein in having a conserved QALGGH motif in the putative DNA-contacting domain and in the presence of long spacers of varied lengths between zinc fingers (Takatsuji, 1999). *TAZ1* codes for a 444–amino acid polypeptide that contains three zinc finger domains. Other members of this class, such as ZPT2-1 and ZPT2-2, have been shown to interact with two tandem but separated core DNA sequences in a manner that is dependent on the length of the spacer between the two core sites (Takatsuji and Matsumoto, 1996). Existing homologies between these two zinc finger proteins and TAZ1 suggest that TAZ1 is a DNA binding protein. This notion is supported further by the subcellular localization of TAZ1 in the nucleus.

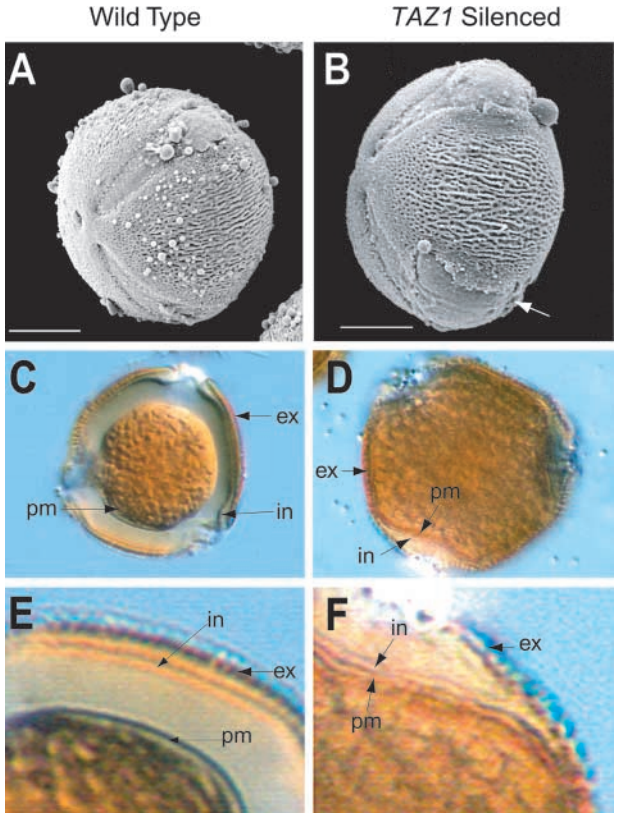

Figure 10. Characterization of the Pollen Wall.

(A) and **(B)** Scanning electron micrographs of pollen from mature buds (Bl 65 mm) of wild-type and *TAZ1*-silenced plants. The place of anomaly in pollen wall formation accompanying a bulging germination pore is marked with an arrow in **(B)**. Bars = 10 μm.
(C) Pollen grains from wild-type anthers in a 20% glycerol solution.
(D) Pollen grains from *TAZ1* cosuppression lines in 50% glycerol.
(E) and **(F)** Magnified views of **(C)** and **(D)**, respectively.
ex, exine; in, intine; pm, plasma membrane.

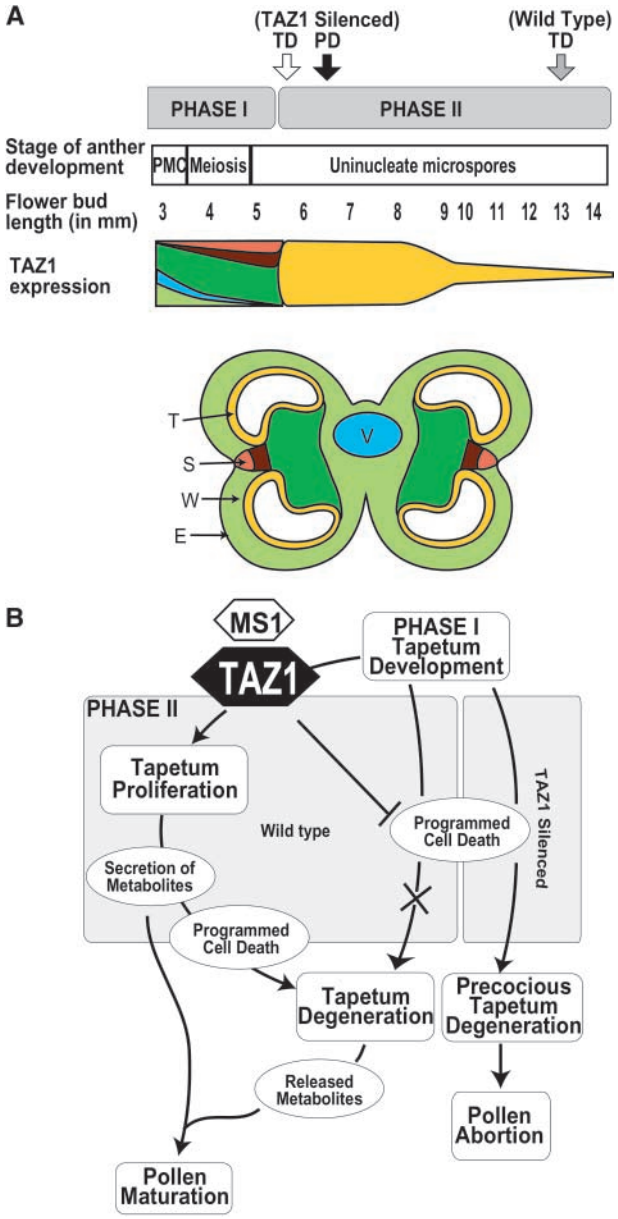

Figure 11. Summary of *TAZ1* Expression and Proposed Model of Its Role.

(A) Temporal and spatial regulation of *TAZ1* mRNA during anther development. Summary of the RNA gel blot and in situ hybridization experiments shown in Figures 1 and 2. The thickness of the colored bar represents the relative amount of *TAZ1* transcript at a particular stage of anther development, and the different colors correspond to the duration and the anther region in which *TAZ1* expression was localized. Early stages of anther development, along with the corresponding bud lengths and the hypothetical phases (I and II) of tapetum development, are shown. The initiation of tapetum and pollen degeneration in *TAZ1*-silenced and wild-type plants are marked with downward arrows. E, epidermis; PD, pollen degeneration; PMC, pollen mother cells; S, stomium; T, tapetum; TD, tapetum degeneration; V, vascular bundle; W, wall.

The expression pattern of *TAZ1* is summarized along with the stages of anther development in Figure 11A. Based on the spatial and temporal distribution of its transcripts, the expression of *TAZ1* consists of two distinct phases. In phase I, it is expressed in all of the cell types of the anther except for the tapetum and sporogenous cells; in phase II, *TAZ1* expression is localized exclusively in the tapetum.

During phase I of anther development, which is represented by flower buds up to Bl 5 to 6 mm in petunia, anther primordia differentiate into specific cells and tissues, the morphology of the anther is established, and sporogenous cells undergo meiosis to produce haploid microspores (Goldberg et al., 1993). After their differentiation, tapetal cells divide mostly anticlinally in petunia, forming a single layer of columnar cells that lines the anther locule at the end of meiosis. During meiosis, tapetal cells also undergo nuclear divisions, resulting in an overall increase in DNA content from 2 to 4C (during meiotic prophase) to 7 to 8C by the end of meiosis (Liu et al., 1987). Because they are intensely involved in the replication of DNA, tapetal cells have been shown to lack poly(A) RNA. For this reason, the tapetum has been suggested to play no active role in the nutrition of microsporocytes before and during meiosis (Raghavan, 1989). In the sequence of developmental events, the first known contribution of the tapetum to the developing microspores is the release of callase, which frees individual microspores by digesting the callose wall around tetrads (Frankel et al., 1969; Stieglitz and Stern, 1973). In *TAZ1*-silenced lines, we found no apparent anomaly in the development of tissues (e.g., connective, vascular tissue, anther wall, and stomium) that expressed *TAZ1* during the premeiotic phase of anther development. Moreover, none of the developmental events mentioned above were affected by *TAZ1* silencing. The microsporocytes underwent normal meiosis and produced pollen tetrads. A single-layered tapetum also was observed at the tetrad stage, and individual microspores were separated, suggesting adequate and timely production of callase from the tapetum. The absence of *TAZ1* transcripts in both phase I and phase II anthers of *TAZ1*-silenced lines was confirmed by RNA gel blot analysis. Therefore, the apparently wild-type-like phenotype of premeiotic anthers in *TAZ1*-silenced lines suggests either that the function of *TAZ1* is redundant with those of other genes or that this gene has no function in cell types other than the tapetum. A possible translational block as a result of the 5′ untranslated region of *TAZ1*, which was suggested by promoter-GUS experiments, could account for the absence of a phenotype during phase I.

(B) Proposed model of the role of TAZ1 in tapetum development. The placement of MS1 with TAZ1 is based solely on the similarity of their respective mutant and silencing phenotypes.

From the time of completion of meiosis in the microsporocytes to the stage at which the tapetum begins to disintegrate, tapetal cells are characterized by the highest rates of transcriptional activity of all of the cell types in the anther (Raghavan, 1981, 1989). During this phase (phase II), *TAZ1* was expressed exclusively in the tapetum. The beginning of phase II also was characterized by the rapid multiplication of tapetal cells. As a result of both periclinal and anticlinal divisions, the number of tapetal layers increased from one (monoseriate) at the tetrad stage to at least three (triseriate) by the Bl 6-mm stage. After Bl 6 mm and at least until Bl 13 mm, the size of individual tapetal cells increased nearly twofold to fivefold; however, because of the gradual degeneration of the inner layers, the tapetum was left with only a single layer of cells by the Bl 13-mm stage. By contrast, the phase of tapetum proliferation was completely absent in *TAZ1*-silenced lines. Soon after the release of individual microspores from tetrads, tapetal cells began to shrink and collapse, and by the Bl 9-mm stage, the tapetum was represented by a single layer of darkly stained collapsed cells in the anthers of *TAZ1*-silenced lines.

The importance of the tapetum in pollen development has been demonstrated using the "genetic laser" technique (Koltunow et al., 1990; Mariani et al., 1990). In these experiments, selective destruction of the tapetum caused by the expression of cytotoxin genes under the control of a tapetum-specific *TA-29* promoter resulted in the complete degeneration of pollen grains in tobacco. Recently, mutation in a tapetum-specific Arabidopsis gene, *MALE STERILE1* (*MS1*), was demonstrated to cause precocious degeneration of the tapetum and complete male sterility (Wilson et al., 2001). MS1 is a plant homeodomain protein finger protein that lacks any apparent similarity to TAZ1. The reported expression pattern of the *MS1* gene was similar to that of *TAZ1* during phase II. As with *TAZ1*, the accumulation of *MS1* transcripts begins only after male meiosis is complete and individual microspores have separated from the tetrads. Furthermore, both *TAZ1* and *MS1* code for putative transcription factors. The similarity in the expression pattern and the phenotypes resulting from the lack of their respective expression suggest that both *TAZ1* and *MS1* could serve as components of the regulatory mechanism that controls the postmeiotic phase of tapetum development.

Based on the data presented here, we propose a hypothesis for the possible role of *TAZ1* (Figure 11B). According to this model, both constructive and degenerative forces act simultaneously during phase II of tapetum development. The degenerative forces are governed by the programmed cell death mechanism, whereas TAZ1, along with MS1, forms an essential component of the constructive forces that cause tapetal cells to proliferate, allowing them to play their role in sustaining pollen development. In the absence of TAZ1 (in silenced lines), however, the programmed cell death mechanism proceeds unchecked and causes precocious degeneration of the tapetum. The absence of the cell proliferation and synthesis phase in the *TAZ1*-minus development deprives the developing microspores of nutrients, resulting in their premature abortion.

METHODS

Cloning of the *TAZ1* Gene and Its Promoter

The *TAZ1* gene was isolated by screening a genomic DNA library of *Petunia hybrida* var Mitchell in EMBL3 vector. Subsequently, a 2.2-kb EcoRI fragment containing a 1991-bp promoter region upstream of the ATG initiation codon and 209 bp of the coding region was subcloned in pBluescript SK+ (Stratagene, La Jolla, CA) and sequenced. The sequence was submitted to DDBJ.

Plasmid Construction

TAZ1::*GUS*

The *TAZ1*::*GUS* reporter gene was constructed by fusing a 2.0-kb upstream fragment (from the 2.2-kb EcoRI fragment mentioned above) of the *TAZ1* gene to the β-glucuronidase (GUS) coding sequence. A 20-base oligonucleotide (5′-GATCTATATGTCGACATA-TA-3′; the SalI site is underlined) was self-hybridized and introduced as a linker into the BglI site that was located 13 bp downstream of the *TAZ1* translation initiation codon. Thereafter, the 2012-bp HindIII-SalI fragment (HindIII is of pBluescript SK+; SalI is a restriction site introduced as part of the linker) was spliced into the same sites of pUCAP (van Engelen et al., 1995), placing it 5′ to the GUS gene that was cloned already in SmaI and SacI sites of this vector. This synthetic gene containing *TAZ1*(promoter)::*GUS*::*Tnos* was excised from pUCAP using AscI and PacI and was inserted into pBINPLUS (van Engelen et al., 1995) using the same set of enzymes.

35S::*TAZ1*

A SalI-NotI fragment containing the complete *TAZ1* cDNA (*cTAZ1*) sequence (Kobayashi et al., 1998) was excised from pSPORT vector using KpnI and SacI. This fragment was inserted between the 35S promoter of *Cauliflower mosaic virus* (*CaMV35S*) and the nopaline synthase (Nos) terminator, which were cloned already in the SalI and EcoRI sites, respectively, of pUCAP (van Engelen et al., 1995). This synthetic gene containing *CaMV35S*::*cTAZ1*::*Tnos* was excised from pUCAP using AscI and PacI and was inserted into pBINPLUS (van Engelen et al., 1995) using the same set of enzymes.

35S::*GFP*::*TAZ1*

The complete *TAZ1* coding sequence was amplified by PCR using two oligonucleotide primers, 5′-ACTAGGGCCCATGGTTGATTATCA-AGATCTTCAAGTTGGG-3′ and 5′-ACTAGGGCCCTTAAATTGGAA-AAAATGTAAAATACTGATGATCACGG-3′. The underlined regions denote XmaI sites that were added to both primers to facilitate the cloning of amplified DNA. After digestion with XmaI, the coding re-

gion of *TAZ1* was ligated to the XmaI-linearized psGFPcs vector (Jiang et al., 2001), placing it between the green fluorescent protein (GFP) coding sequence and the Nos terminator.

Plant Transformation

The *TAZ1*::*GUS* and *35S*::*TAZ1*::*NosT* chimeric genes were introduced into petunia plants by *Agrobacterium tumefaciens* (GV3101)–mediated transformation (Jorgensen et al., 1996). After regeneration on selective medium, transformed petunia lines were checked for the presence of the transgene by PCR of the neomycin phosphotransferase II sequence and were transferred to a greenhouse.

Transient expression of the *35S*::*GFP*::*TAZ1* gene in onion peel cells was performed as described by Jiang et al. (2001). The fluorescence of sGFP fusion proteins was observed with a confocal microscope (Bio-Rad Laboratories, Hercules, CA), and the resulting digital micrographs were assembled using Photoshop software (Adobe Systems, San Jose, CA).

RNA Gel Blot Analysis

Total cellular RNA from petunia leaves and anthers was isolated using the procedure developed by Logemann et al. (1987) and stored at −70°C in 95% ethanol. Aliquots (10 μg) of RNA were separated on 1.2% agarose gels containing 0.4 M formaldehyde and transferred to Gene Screen membranes (DuPont–New England Nuclear Life Science Products, Boston, MA). A digoxigenin-labeled antisense RNA corresponding to the 1172-bp 3′ terminal region (3′ of the EcoRI site at position 717) of *cTAZ1*, prepared according to the manufacturer's instructions (Boehringer Mannheim), was used as a probe. Hybridization of blots and detection of chemiluminescence also were performed according to the Boehringer Mannheim protocol.

In Situ Hybridization

In situ hybridization was essentially carried out by using Ribomap kit and Discovery automatic staining module (Ventana Medical Systems, Tucson, AZ according to manufacturer's instructions). The excised anthers were fixed in formaldehyde 10%, acetic acid 5%, and ethanol 50% for four days at 4°C. The *cTAZ1* was digested with EcoRI and SpeI. The 491-, 243-, 263-, 167-, 154-, 434-, and 172-bp fragments generated were cloned in pBluescript SK+. Both antisense and sense probes from all of the c*TAZ1* fragments were synthesized using the SP6/T7 digoxigenin RNA labeling kit (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instructions. The sense and antisense digoxigenin-labeled RNAs were pooled and used for hybridization with the fixed anther sections at 60°C for 6 h in Ribohyb hybridization solution (Ventana Medical Systems). After hybridization, sections were washed in 0.1 × SSC (three times for 6 min) at 70°C. Detection of hybrids was performed using the digoxigenin nucleic acid detection kit (Boehringer Mannheim), according to the manufacturer's instructions. Detection of hybrids with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate 4-toluidinium salt was performed at 20°C for 16 h in the dark. Sections were dehydrated through an ethanol series (30, 50, 70, 90, and 100% ethanol for 2 min each) and washed twice for 5 min in xylene before mounting in Malinol mounting medium (Muto Pure Chemicals, Tokyo, Japan).

Cultivation and Observation of Pollen Tubes

For in vitro culture, pollen grains from a single anther were dusted onto a petri dish containing solid pollen germination medium [0.05% $Ca(NO_3)_2 \cdot 4H_2O$, 0.01% H_3BO_3, 5% Suc, and 0.15% gellan gum] according to the protocol described by Higashiyama (2000). Plates were incubated for 14 h at 25°C and then analyzed and photographed using the phase-contrast option in a DMR microscope (Leica Microscopy Systems, Wetzlar, Germany).

For semi-in vivo germination, pollen grains of either wild-type or *TAZ1*-silenced plants were smeared onto the stigmas of wild-type pistils. The styles were cut (1 cm from the top) at 15 min after pollination and placed onto the solid pollen germination medium. To compensate for the low pollen count in *TAZ1*-silenced lines, three anthers were used to pollinate a single stigma, instead of one in the case of the wild type. This experiment was performed in triplicate. After 14 h, the plates were examined using the dark-field settings of the DMR microscope.

Detection of Flavonols

Flavonol staining was performed essentially as described (Ylstra et al., 1994). Pollen from freshly dissected anthers was placed for 2 h in a saturated solution (<0.5%, w/v) of diphenylborinic acid ethanolamine ester (Aldrich) with 0.01% Triton X-100 and 10% glycerol. After washing with a solution of 10% glycerol, the pollen was visualized and photographed under UV light using the DMR microscope.

Pollen Plasmolysis

The anthers were pressed open (by gently pressing the cover glass to release the pollen grains) in 20-μL hypertonic solutions (13 to 50% glycerol) on glass slides. After 5 min of incubation at room temperature, the solutions were covered with glass cover slips and photographed using the Nomarski optics (interference microscopy) option of the DMR microscope.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

Accession Number

The DDBJ accession number for *TAZ1* is AB063169.

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